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Introduction:

The majority of our present chemotherapeutic agents only kill cells effectively when they are proliferating; this may explain why these agents have been of such limited success in patients. In contrast to these ineffective agents, we have chemically modified a plant toxin, Thapsigargin (TG), to produce primary amine-containing analogs that are potent, cell proliferation independent, inducers of apoptosis in prostate cancer cells. These TG-analogs, however, are not prostate cancer-specific cytotoxins. Therefore, the hypothesis of this proposal is that a potent TG analog can be converted to inactive prodrugs by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). In this way the inactive prodrugs can be efficiently converted back to active killing drugs only by the enzymatic activity of PSMA. Since PSMA is expressed in high levels only by prostate cancer cells and not by normal cells, this should allow specific targeting of the TG-analog's killing ability to prostate cancer cells.

Two enzymatic activities for PSMA have been described: an N-acetyl-a linked acid dipeptidase (NAALADase) activity and a pteroyl poly-g-glutamyl carboxypeptidase (folate hydrolase) activity. On the basis of preliminary data, the ideal TG prodrug should consist of either an aspartate or glutamate containing TG analog coupled via to a peptide containing a series of a- and g-linked glutamates and ending in an alpha-linked aspartyl-glutamate "cap". This substrate would be readily cleaved by PSMA but would be stable to hydrolysis by gamma-glutamyl hydrolase (GGH) present in serum and extracellular fluid of some normal tissue types.

Therefore, the specific aims of the study are: (1) to synthesize a series of aspartate or glutamate containing TG analog and characterize their cytotoxicity against human prostate cancer cell lines (2) to synthesize prodrugs by chemically linking the aspartyl and glutamyl containing TG analogs to peptide substrates that require hydrolysis by both the NAALADase activity and folate hydrolase of PSMA. These prodrugs will be assayed for in vitro cytotoxicity against PSMA-producing and non-producing cell lines to determine which of these prodrugs is the best lead compound for future in vivo animal studies; (3) to determine the in vivo efficacy and toxicity of the best (i.e. lead) PSMA prodrug (based upon comparison of the in vitro potency and specificity) against PSMA-producing human prostate cancer xenografts; (4) to develop a molecular model of the PSMA catalytic site and compare binding of peptide substrates tested to date in order to predict optimal PSMA peptide substrate.

BODY:

PSMA is a 100 kDa prostate epithelial cell type II transmembrane glycoprotein that was originally isolated from a cDNA library from the androgen responsive LNCaP human prostate cancer cell line. Using a series of monoclonal antibodies, several groups have characterized PSMA expression in various normal and tumor tissues. These studies demonstrated that PSMA is expressed in high levels by both normal and malignant prostate tissue. Low-level expression was only seen in the lumen of the small intestine and the proximal tubules of the kidney. In addition, while normal vascular endothelial cells are PSMA negative, endothelial cells of the tumor vasculature stain positive for PSMA in a large number of tumor types, while the tumor cells themselves do not express PSMA.

In summary, the aforementioned studies highlight the characteristics of PSMA that make it a suitable target for prostate specific therapy. PSMA expression is highly restricted to prostate tissue with strongest expression in both primary and metastatic prostate cancers. The PSMA protein detectable in prostate cancers is an integral membrane protein and therefore has an extracellular domain that is accessible to agents in the extracellular peritumoral fluid making it possible to target this protein with antibodies and prodrugs. A final interesting aspect of PSMA expression is that the PSMA mRNA is upregulated upon androgen withdrawal. In LNCaP cells androgen has been found to downregulate PSMA expression and in patient specimens an increase in immunohistochemically detectable PSMA expression has been observed following androgen ablative therapy. In contrast, PSA expression is downregulated by androgen deprivation. Therefore, PSMA should be readily targetable in the majority of hormone refractory patients because PSMA levels are expected to remain high following androgen ablation.

Two discrete enzymatic functions for PSMA have been described. Initially, Carter et al demonstrated that PSMA possesses the hydrolytic properties of an N-acetylated α -linked acidic dipeptidase (NAALADase). NAALADase is a membrane hydrolase activity that is able to hydrolyze the neuropeptide N-acetyl-L-aspartyl-L-glutamate (NAAG) to yield the neurotransmitter glutamate and N-acetyl-aspartate. In addition to the NAALADase activity, PSMA also functions as a pteroyl poly- γ -glutamyl carboxypeptidase (folate hydrolase). PSMA exhibits exopeptidase activity and is able to progressively hydrolyze γ -glutamyl linkages of both poly- γ -glutamated folates and methotrexate analogs with varying length glutamate chains. In our proposal, we outline an approach that would take advantage of both the prostate specific expression of the PSMA protein in men and its unique NAALADase and folate hydrolase activities.

A successful PSMA activated prodrug must pass three critical tests. The prodrug must be cleaved by PSMA, it must not be toxic to PSMA-negative cells and its toxicity to PSMA positive cells must be secondary to activation specifically by PSMA. In order to rapidly screen a larger number of prodrugs for PSMA activity and specificity, we previously synthesized a series of analogs based on methotrexate consisting of the pteridine ring-para-aminobenzoic acid (APA) portion of methotrexate coupled to a variety of peptides. This strategy was utilized for several reasons. The coupling of APA to the N-terminal amine of gamma-linked polyglutamates does not inhibit sequential PSMA hydrolysis. The chemistry to produce these analogs has already been described and the large quantities of the inexpensive APA precursor are available. The APA molecule has an absorbance at 340 nm and hydrolysis of prodrugs can be readily followed by HPLC analysis. Finally, APA-Glu (i.e. methotrexate) is cytotoxic at nanomolar concentrations and therefore activation of prodrugs in vitro can be easily assessed using growth inhibition and clonogenic survival assays. This strategy, therefore, was used to identify peptides that were selectively hydrolyzed by PSMA while at the same time remaining stable to hydrolysis in human plasma.

These studies with methotrexate-based peptides have delineated the limited range of substrates that can be hydrolyzed by PSMA. The only alpha-linked methotrexate substrate (i.e. substrate for PSMA's NAALADase activity) that was hydrolyzed by PSMA was APA-Asp-Glu. This substrate was also stable to hydrolysis by gamma-glutamyl hydrolase (GGH) and other protease activity present in the serum, table 1. These previous studies also demonstrated the enhanced ability of PSMA to hydrolyze a variety of γ -linked substrates. These γ -linked substrates, however, were not specific for PSMA and were readily hydrolyzed in human plasma. On this basis, we reasoned that the ideal substrate would take advantage of the dual ability of PSMA to

hydrolyze certain alpha and gamma linkages between aspartyl and glutamyl residues. Thus, the ideal substrate should incorporate the specificity of the α -linkage with the enhanced efficiency of the γ -linkage. The longer length, negatively charged, substrates would serve two additional purposes: first, they help to make the highly lipophilic more toxins like TG analogs more water soluble; second, the highly charged prodrug will be less likely to cross the plasma membrane, further limiting non-specific cytotoxicity, figure 1.

Table 1. Hydrolysis of PSMA-substrates by purified PSMA and stability in plasma

Substrate	% PSMA ² Hydrolysis		18 h Incubation Human Plasma		18 h Incubation Mouse Plasma
	4 h	24h	% APA-Glu or Asp	% Prodrug Remaining ¹	% APA-Glu or Asp
α-linked					
APA-Asp-Glu	0	62	1	99	ND
APA-Glu-Glu	0	0	ND	ND	ND
γ-linked					
APA-Glu-Glu-Glu-Glu-Asp	18	86	9	20	72
APA-Glu-Glu-Glu-Glu-Gln	48	92	6	26	62
APA-Glu-Glu-Glu-Glu-Glu	50	96	11	11	78

¹% Prodrug Remaining equals peak area of starting material/total peak area (sum of prodrug and intermediate peaks). ² % complete hydrolysis to APA-Asp or APA-Glu by purified PSMA. ND= not done

Using this rationale, additional substrates were synthesized in which a PSMA-hydrolyzable α -linked dipeptide "cap" is introduced that is not a substrate for GGH in order to produce more specific PSMA substrates. One of these substrates APA-Glu*Glu*Glu*Asp-Glu was a less efficient PSMA substrate but showed enhanced stability in human serum. A second substrate APA-Glu*Glu*Glu*Asp-Gln was a poor substrate for PSMA, although it had even more enhanced stability in serum. Finally, a substrate containing two α -linkages and two γ -linkages, APA-Asp-Glu*Glu*Asp-Glu was a better PSMA substrate and was completely stable to hydrolysis in human and mouse plasma, table 1. These combination alpha- and gamma-linked PSMA substrates possess the best combination of efficiency and specificity and these substrates will therefore be used to create prodrugs described below.

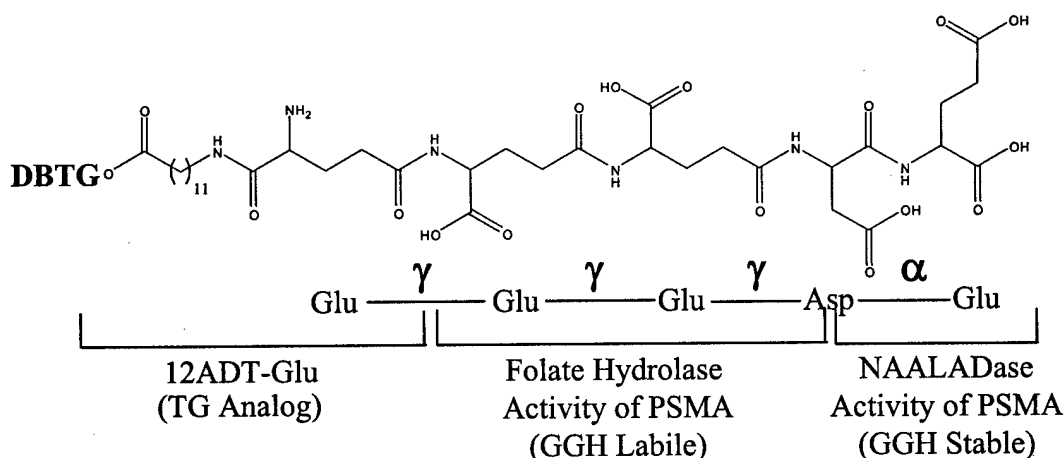
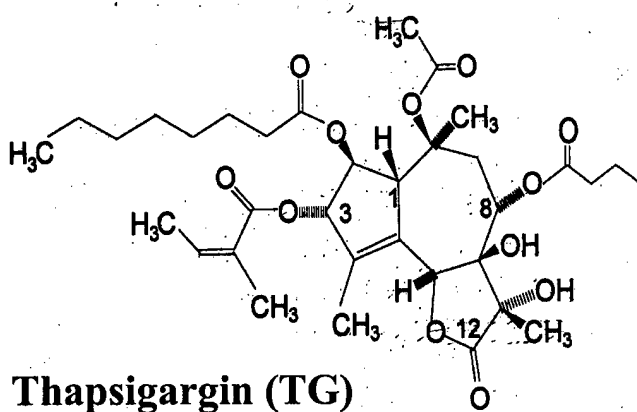


Figure 1. Example of PSMA prodrug containing α - and γ -linked amino acids and TG analog 12ADT-Glu.

Thapsigargin (TG) is a sesquiterpene γ -lactone isolated from the root of the umbelliferous plant, *Thapsia garganica*, figure 2. TG has been shown to increase intracellular Ca^{2+} and induce programmed cell death in prostate cancer cell lines as well as a host of other normal and malignant cell types. TG induces programmed cell death of all rapidly proliferating prostate cancer cell lines. Unlike standard antiproliferative agents such as 5-FU or doxorubicin, TG can also induce apoptosis in non-proliferating, G_0 arrested prostate cancer cells. TG represents an excellent choice for treatment of prostate cancer because of its ability to kill these cells in a proliferation-independent manner. This ability to induce apoptosis in a proliferation-independent manner is particularly advantageous for the treatment of prostate cancer since these tumors have been demonstrated to have a remarkably low rate of proliferation (i.e. $< 5\%$ cells proliferating/day). Unfortunately, while TG is highly effective in inducing the proliferation independent programmed cell death of androgen independent prostate cancer cells, it is not cell type specific and is sparingly water soluble due to its high lipophilicity. In order to target TG's cytotoxicity specifically to prostate cancer cells systemically, TG has been chemically modified to produce a primary amine containing analog that can be coupled to a water-soluble peptide carrier. This modification involves the introduction of a primary amine containing side chain into the TG molecule that can be coupled via a peptide bond to the carboxyl group of amino acid. Such a TG analog can be coupled to the alpha (Asp or Glu), beta (Asp) or gamma (Glu) carboxyl of Asp or Glu residues to produce prodrugs that can be targeted specifically to metastatic deposits of androgen independent prostate cancer producing enzymatically active PSMA, figure 3.



Progress over the 2003-2004 funding period.

In **task 1** of the proposal we proposed the synthesis of a series of TG analogs that could be chemically linked to PSMA specific peptides. Because PSMA is an exopeptidase, hydrolytic processing of any prodrug will result in an end product consisting of a cytotoxin coupled to an acidic amino acid, most likely glutamate or aspartate. Therefore, while a TG analog can be potentially targeted using this prodrug approach, preferred analogs would be those that incorporate glutamic or aspartic acid into their structure and still maintain their cytotoxicity. Previously we synthesized the analogs 12ADT-Glu and 12ADT-Asp and demonstrated that they were potent cytotoxins with IC₅₀ values against cancer cell lines of < 100 nM.

The goal of Task 2 of the proposal was to synthesize prodrugs by chemically linking the TG analogs to peptide substrates that require hydrolysis by either NAALADase activity alone or combined NAALADase and folate hydrolase activity of PSMA. Over the 2002-2003 funding year we synthesized two putative PSMA prodrugs, 12ADT*Asp-Glu and 12ADT*Asp-Glu*Glu*Asp-Glu, and characterized each on the basis of PSMA

hydrolysis, plasma stability and selective cytotoxicity to PSMA producing cells. Over the 2003-2004 funding period we have synthesized additional prodrugs that were similarly characterized, figure 3.

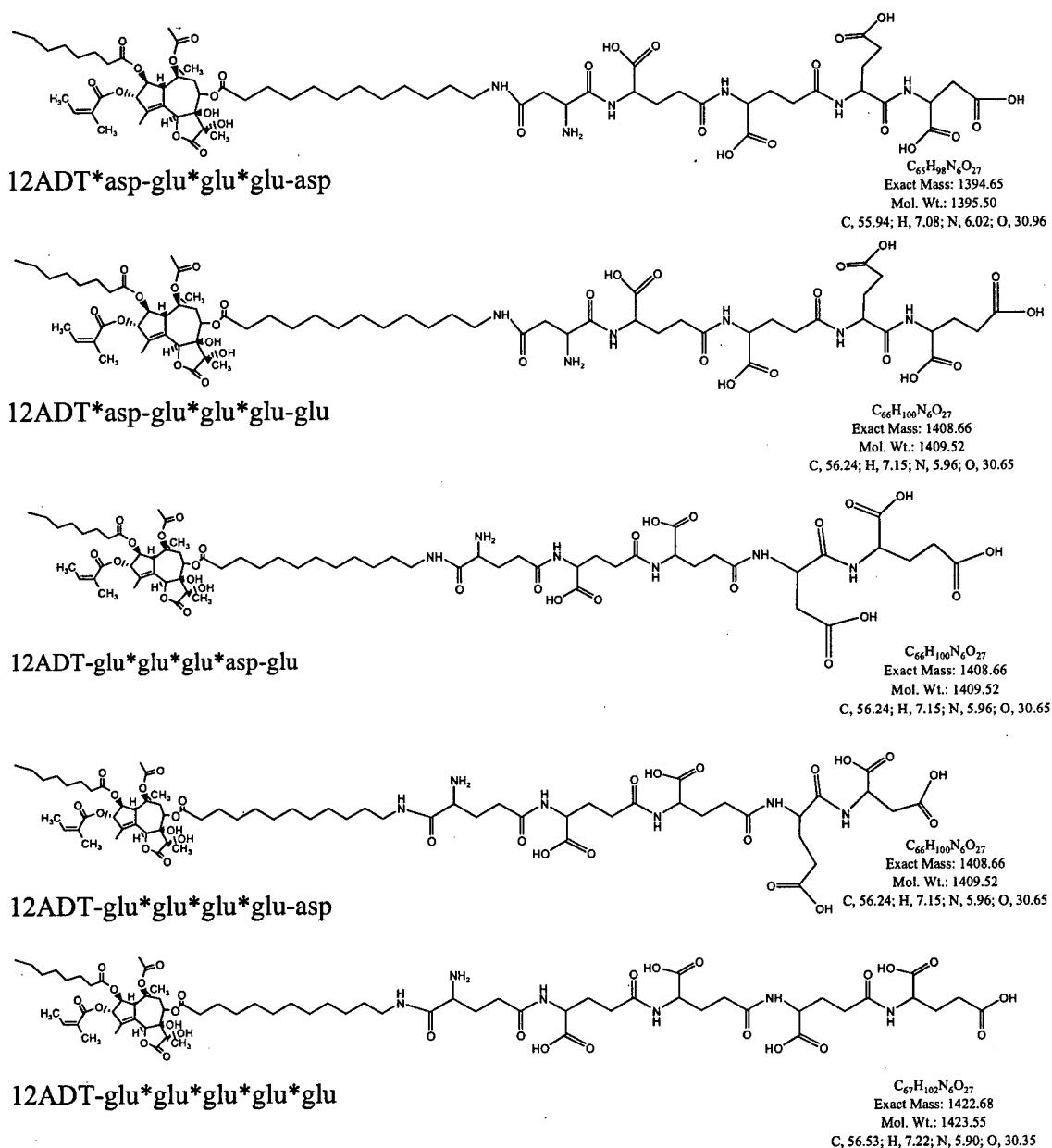


Figure 3. Chemical structures of additional putative PSMA-activated TG prodrugs

To assess PSMA hydrolysis, prodrugs were incubated with PSMA and hydrolysis products were determined by HPLC analysis. Peak identities were confirmed by mass spectroscopy. Amm3, Amm4 and Amm8 were not significantly hydrolyzed after 90 hr incubation with PSMA. For analogs Amm1 and Amm7 the prodrug is hydrolyzed to intermediates but minimal hydrolysis to product occurred. The expected "gold standard sequence", 12Adt-glu*glu*glu*glu*glu (Amm5) was rapidly hydrolyzed to 12Adt-glu*glu but no further hydrolysis to 12ADT-Glu was observed even after 70 h incubation with PSMA. To further assess specificity, all prodrugs were incubated in human plasma for 48 h at which time no significant proteolysis of any of the analogs was observed.

In clonal survival assays 12Adt*asp-glu (Amm2) was cytotoxic to PSMA producing C42B cells and this cytotoxicity was inhibited by 2-(phosphonomethyl)pentanedioic acid (2-PMPA), a competitive inhibitor of PSMA's enzymatic activity. These results demonstrated that increased toxicity of Amm2 was dependent on presence of enzymatically active PSMA in this cell line. The toxicity of all prodrugs were further studied in non-PSMA producing TSU and compared to the PSMA-producing LNCaP cell line. All of the tested prodrugs showed significant reduction in LNCaP cell growth in these assays over the dose range tested. None of the prodrugs, however, were as effective as the active drugs alone (figure 4, table 2). All prodrugs were less potent against non-PSMA expressing TSU cells (figure 4, table 2). These data depict that all prodrugs are relatively "inactive" in the absence of PSMA. The IC₅₀ values (prodrug concentration required to inhibit colony survival by 50%) were calculated (table 2). IC₅₀ values against TSU were in the micromolar range while values against LNCaP were in the nanomolar range. Overall the prodrugs were 15-35 fold more active in vitro against PSMA producing cells, table 2.

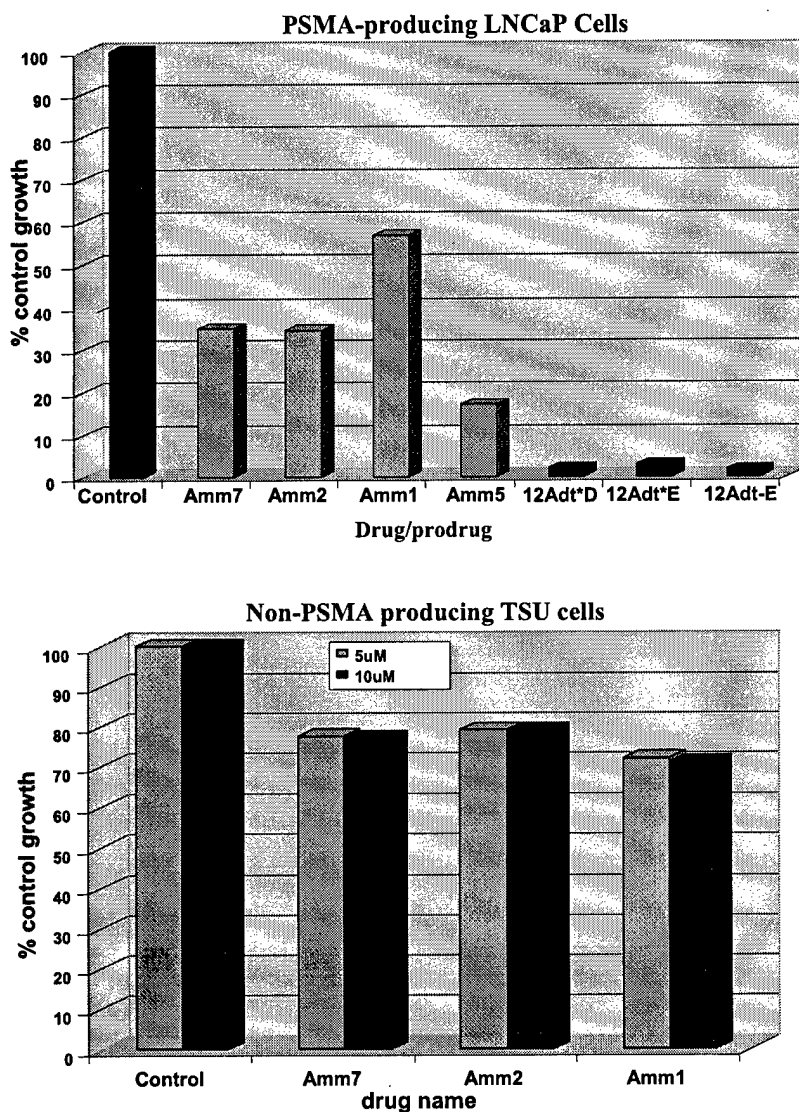


Figure 4. Cytotoxicity of PSMA-activated prodrugs against PSMA-producing LNCaP (top panel) and non-PSMA producing TSU (bottom panel) cell lines. Cells were exposed to prodrug for 7 days and then MTT assay performed to determine number of viable cells per treatment group. LNCaP cells exposed to 1 μ M prodrug and TSU exposed to either 5 or 10 μ M.

Table 2. IC₅₀ values for selected prodrugs against PSMA-producing LNCaP cells and non-PSMA producing TSU cells in clonal survival assays.

Prodrug/drug	IC ₅₀ Values (nM)		
	TSU	LNCaP	Fold difference
12ADT*Asp	160±61	60±15	2.7
12ADT-Glu	82± 4	65± 15	1.3
12ADT*Glu	60 ± 19	60 ± 6	1
AMM1: 12Adt*asp-glu*glu*asp-glu	10816 ± 2141	717 ± 32	15
AMM2: 12Adt*asp-glu	6602 ± 1400	280 ± 30	24
AMM5: 12Adt-glu*glu*glu*glu*glu	8545 ± 444	240 ± 40	35

After having established that all prodrugs show diminished potency against non-PSMA producing cell lines, it was critical to determine whether this decrease was due to the absence of PSMA and poor cell penetration by the peptide-based prodrug. Alternatively, the presence of the peptide carrier is not sufficient to prevent cell penetration and the decreased potency in non-PSMA producing cells, therefore is due to both lack of PSMA hydrolysis of the prodrug and the poor inhibition of the SERCA pump by intracellular prodrug. To address these possibilities, microsomes were isolated by differential centrifugation from rabbit muscle, which are very rich in SERCA. An enzyme-coupled assay utilizing auxiliary and indicator reactions was employed to quantify Ca²⁺ ATPase activity in microsomes. Ca²⁺ ATPase activity in isolated SERCA decreased with increase in inhibitor (drug/prodrug/TG) concentration. The IC₅₀ values expressed in nM/mg SR protein (inhibitor concentration required to decrease CaATPase activity to 50%) were determined for various prodrug analogues and normalized to TG or its drug analog 12Adt*asp (table 3). The longer prodrugs exemplified by 12Adt-glu*glu*glu* asp- glu (Amm7) and 12Adt*asp-glu*glu* asp- glu (Amm1) had the lowest potency against SERCA. 12Adt*asp-glu (Amm2) showed higher potency than the pentamers but lower potency than the drug 12Adt*asp. The ultimate drug 12Adt*asp was equipotent to the natural SERCA inhibitor TG. Although IC₅₀ values increased with chain length in the SERCA assays, the magnitude (~6nM vs. 25nM) is small compared to the difference between IC₅₀ values in clonal assays in non-PSMA and PSMA producing lines (nM vs. uM range) (table 2). These results suggest that the fold differences in potency of these prodrugs are due to prodrug processing by PSMA-producing cells. In contrast, the peptide prevents non-specific cell penetration and therefore less cytotoxicity against non-PSMA producing cells.

Table 3. Inhibition of SERCA pump by TG and PSMA-activated TG prodrugs

Drug	IC ₅₀ nM/mg SR protein	IC ₅₀ Relative to TG	IC ₅₀ Relative to 12Adt*Asp
TG	6.6 +/- 2.3	1	0.9
12Adt*Asp	7.5 +/- 2.2	1.2	1
Amm1	24.3 +/- 1.9	3.8	3.2
Amm2	15.3 +/- 1.7	2.4	2
Amm7	26.1 +/- 3.7	4.1	3.5

KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized a series of PSMA-activated thapsigargin peptide prodrugs.
2. Demonstrated that several of the TG prodrugs are efficiently hydrolyzed by PSMA.
3. Demonstrated that these PSMA-activated TG prodrugs are stable to hydrolysis in human and mouse plasma
4. Determined cytotoxicity of these prodrugs against PSMA-producing and PSMA non-producing human cancer cell lines and found ~15-35-fold difference in cytotoxicity in cells that produced PSMA vs. cells that did not.
5. Synthesized sufficient quantities of lead PSMA-activated prodrugs to initiate in vivo toxicology and antitumor efficacy studies.

REPORTABLE OUTCOMES:**Presentations:**

“Enzyme activation of Thapsigargin Prodrugs by Prostate-Specific Membrane Antigen as Targeted Therapy for Prostate Cancer”. Poster presentation at Fellow’s Research Day, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD, 2002.

Graduate student Anastasia Mhaka winner of prize for “Best Translational Research Presentation”.

“Enzyme activation of Prodrugs by Prostate-Specific Membrane Antigen”. Poster presentation at EORTC/American Association of Cancer Research (AACR) meeting, Frankfurt, Germany, 2003

“Targeted Activation of Cytotoxic Prodrugs/Protoxins by Prostate-Specific Proteases” The Brady Urologic Institute Thursday Evening Seminar Series, Johns Hopkins University, Baltimore, MD, 2003.

Manuscripts and Abstracts:

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2. **Mhaka, A.,** Janssen, S., Rosen, M., **Denmeade, S.R.** Enzyme activation of Prodrugs by Prostate-Specific Membrane Antigen. *European J Cancer* 38, Suppl 7:S36, 2002.
3. **Denmeade, S.R.,** Sokoll, L.J., Dalrymple, S., Rosen, D.M., Gady, A.M., Bruzek, D., Ricklis, R.M., Isaacs, J.T. Dissociation Between Androgen Responsiveness for Malignant Growth vs. Expression of Prostate Specific Differentiation Markers PSA, hK2 and PSMA in Human Prostate Cancer Models. *Prostate*, 54:249-257, 2003.

4. **Denmeade, S.R.**, Jakobsen, C., Janssen, S., Khan, S.R., Lilja, H., Christensen, S.B. and Isaacs, J.T. Prostate-Specific Antigen (PSA) Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer, J Natl Cancer Inst. 95:990-1000, 2003.
5. **Mhaka A**, Gady AM, Lo K-M, Gillies S, **Denmeade SR**. Use of Methotrexate-Based Peptide Substrates to Characterize the Substrate Specificity of Prostate-Specific Membrane Antigen (PSMA). Manuscript submitted for publication, 2004.

Patent application:

“Prodrug activation by prostate-specific membrane antigen”, Inventors S. Denmeade and J. Isaacs (PI) submitted May 2000.

Employment:

Salary support for Graduate Student Annastasiah Mhaka to carry out experiments outlined in this proposal.

CONCLUSIONS:

Over the 2003 funding period we have completed tasks 1 and 2 as outlined in our original proposal. Over the ensuing year we will synthesize sufficient quantities of our lead prodrugs to complete in vivo toxicology and antitumor efficacy studies as outlined in task 3 of the proposal. In addition, we have recruited a graduate student with expertise in computer modeling. This student will use the model of the PSMA catalytic site we described in our original proposal to evaluate binding of these substrates and the methotrexate analogs we described in an earlier proposal. These studies will serve to validate the utility of this PSMA model in predicting which substrates would potentially be hydrolyzed by PSMA. Once validated, we will then use this model to screen putative additional substrates "in silico" that could then be synthesized and tested in vitro.

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